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# The *Arabidopsis* *SRR1* gene mediates phyB signaling and is required for normal circadian clock function

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Plants possess several photoreceptors to sense the light environment. In *Arabidopsis* cryptochromes and phytochromes play roles in photomorphogenesis and in the light input pathways that synchronize the circadian clock with the external world. We have identified *SRR1* (sensitivity to red light reduced), a gene that plays an important role in phytochrome B (phyB)-mediated light signaling. The recessive *srr1* null allele and *phyB* mutants display a number of similar phenotypes indicating that *SRR1* is required for normal phyB signaling. Genetic analysis suggests that *SRR1* works both in the phyB pathway but also independently of phyB. *srr1* mutants are affected in multiple outputs of the circadian clock in continuous light conditions, including leaf movement and expression of the clock components, *CCA1* and *TOC1*. Clock-regulated gene expression is also impaired during day–night cycles and in constant darkness. The circadian phenotypes of *srr1* mutants in all three conditions suggest that *SRR1* activity is required for normal oscillator function. The *SRR1* gene was identified and shown to code for a protein conserved in numerous eukaryotes including mammals and flies, implicating a conserved role for this protein in both the animal and plant kingdoms.

[**Keywords:** Circadian rhythm; light-signaling; phytochrome B; *Arabidopsis thaliana*]

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Most organisms are sensitive to various aspects of their light environment. To anticipate the day–night cycles, several activities are under the control of the circadian clock. Clock-regulated processes are physiologically diverse, including the regulation of body temperature in mammals, conidiation in fungi, petal opening in plants, and eclosion in flies. For example, it is estimated that ~5% of all *Arabidopsis* genes are clock controlled at the transcriptional level, which could account for diurnal regulation of a large number of metabolic pathways (Harmer et al. 2000; Schaffer et al. 2001). Global expression profiling in animals has also revealed a large number of circadian-regulated genes (e.g., see McDonald and Rosbash 2001; Storch et al. 2002). In a few cases it has been demonstrated that a functional clock enhances the fitness of the organism, suggesting an evolutionary advantage for anticipating dawn and dusk (Green et al.

2002). In the simplest model, the circadian clock is composed of three main constituents: an input pathway that conveys resetting information to the oscillator, a central oscillator that generates an ~24-h period and clock-controlled output pathways that translate this into physiological rhythms (Young and Kay 2001). The oscillator is regularly reset by the photoperiod; in addition, light-regulated activities can be restricted by the circadian clock so that a pulse of light in the middle of the night does not have the same effect as the same light cue given during the day (Millar and Kay 1996). Both of these phenomena require that the plant responds to light cues in a time-of-day-specific manner. It is therefore apparent that light perception and circadian regulation are intimately linked (Devlin and Kay 2001).

Molecular genetic analysis of clock-controlled activities has identified several key components of the central oscillator in various organisms, including *Drosophila melanogaster*, *Neurospora crassa*, *Synechococcus elongatus*, *Mus musculus*, and *Arabidopsis thaliana* (Young and Kay 2001). In all those organisms transcriptional–translational negative feedback loops are important elements of the oscillator (Young and Kay 2001). Despite this mechanistic similarity, there is little conservation of the biochemical components of the central oscillator.

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In *Arabidopsis*, for example, three gene products have a proposed role in the circadian pacemaker: TOC1 and two related transcription factors, CCA1 and LHY (Alabadi et al. 2002; Green and Tobin 2002; Mizoguchi et al. 2002). These three proteins are unrelated to clock components of *Neurospora*, mice, or *Drosophila* (Young and Kay 2001). In contrast, the cryptochromes (CRYs) play important, but distinct, circadian functions in plants, flies, and mammals (Devlin and Kay 2001).

Analysis of the light input pathways to the clock reveals that several distinct photoreceptor systems are important for clock entrainment (Devlin and Kay 2001). Melanopsin was recently proposed as a circadian photoreceptor in mammals (Berson et al. 2002). The cryptochrome acts as an input photoreceptor resetting the clock in flies (Emery et al. 2000). In *Arabidopsis*, both cryptochromes and phytochromes mediate light input to the clock (Somers et al. 1998a). The phytochromes (PHY) are a class of red/far-red photoreceptors (Quail 2002b). Red light converts Pr, the red-light-absorbing form of phytochrome, into Pfr, the far-red-light-absorbing form, which is the active conformer for many phytochrome-mediated responses. Pfr can be converted back to Pr by irradiation with far-red light. The C-terminal portion of plant phytochromes is composed of two PAS repeats (PER, Arnt, SIM) and a histidine-kinase-related domain (Quail 2002b). A phytochrome-like protein is also an essential component of the light input pathway to the clock in cyanobacteria (Schmitz et al. 2000). In *Neurospora*, WC-1, a transcription factor with a PAS domain, is thought to act as light receptor to the clock (Froehlich et al. 2002; He et al. 2002). However, because *wc-1* and *wc-2* mutants can be entrained neither by light nor by temperature, WC-1 and the related protein WC-2 can also be classified as critical clock components (Crosthwaite et al. 1997). This illustrates the close association between light input pathways and the central oscillator. WC-1 and WC-2 share little sequence similarity with components of the light input pathway from other organisms. However, they contain PAS domains that are also found in the phytochromes and in the ZTL/LPK/FKF protein family. *Arabidopsis* ZTL/LPK/FKFs have been proposed to act in the light input pathway (Nelson et al. 2000; Somers et al. 2000; Schultz et al. 2001).

Signaling downstream of the CRY and PHY photoreceptors in plants has been extensively studied (Quail 2002a). Relatively few components working downstream of CRY during blue light perception are known at present (Lin 2002). In contrast, a large number of proteins have been proposed to act downstream from phytochromes (Quail 2002b). *Arabidopsis* has five phytochrome genes, *PHYA-PHYE*. *phyA* and *phyB* mutants have particularly obvious phenotypes. *phyA* mutants are blind to far-red light (a light quality found under a canopy of plants), and *phyB* appears to be the major photoreceptor for de-etiolation in red or white light (Quail 2002b). In addition to interconverting between its two spectral forms, light also affects the subcellular localization of phytochromes, their ability to interact with sig-

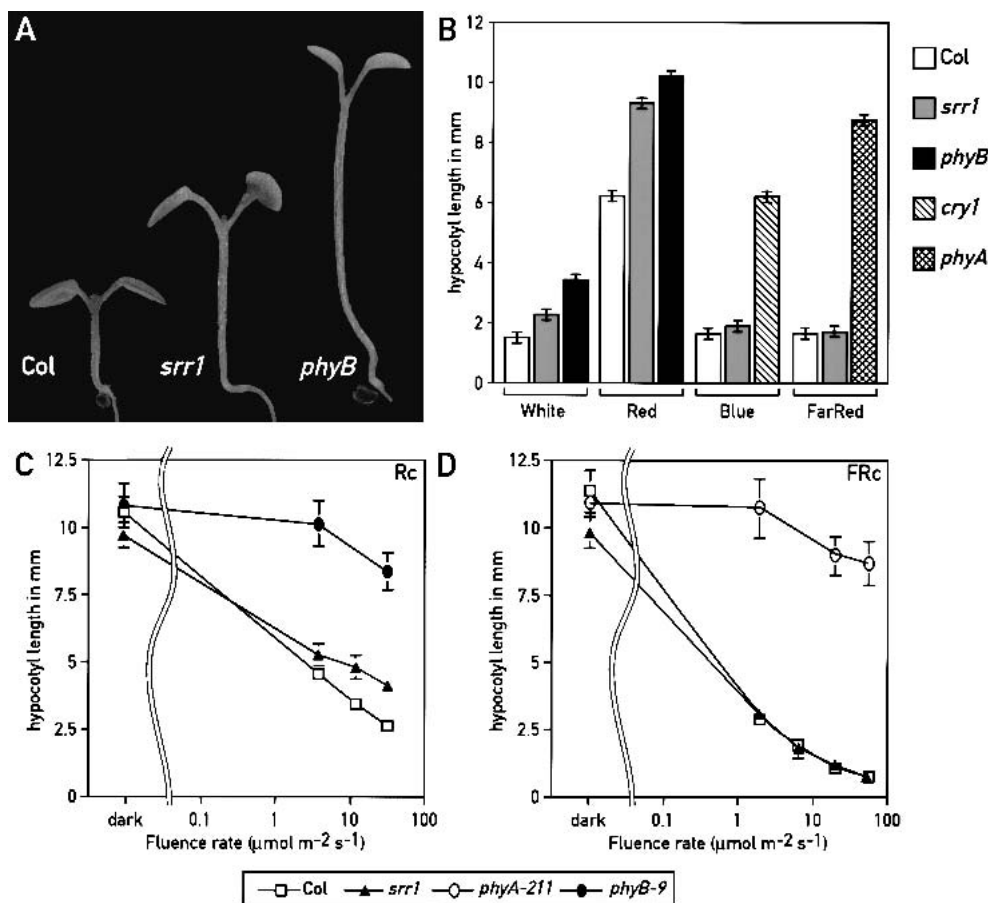
naling partners, and the in vitro kinase activity of phyA (Fankhauser 2000; Quail 2002b). The best understood subpathway involves a direct interaction of phytochrome B with bHLH-domain transcription factors, which results in the activation of multiple genes, including *CCA1* (Martinez-Garcia et al. 2000; Huq and Quail 2002). Light may therefore entrain the plant circadian clock both via transcriptional activation (as it does in *Neurospora crassa* and the mouse) and also via post-translational mechanisms (as in *Drosophila melanogaster*; Somers et al. 2000).

In this report, we describe *srr1*, a new *Arabidopsis* mutant that is altered in multiple outputs of the circadian clock and in phytochrome-B-mediated light signaling. Cloning of the *SRR1* gene reveals that *SRR1* codes for a novel protein with homologs in numerous eukaryotes including mouse and humans. Our studies suggest that *SRR1* might encode a regulator of the circadian clock that is conserved between plants and animals.

## Results

### *The srr1 mutant is impaired in phyB signaling*

In an effort to identify novel components required for proper light-modulated development, we screened for *Arabidopsis* mutants that displayed reduced sensitivity to low intensities of white light. We selected seedlings that had a longer hypocotyl than the wild type in this light regime (Fig. 1A), and identified the *srr1* (sensitivity to red light reduced) mutant. We determined that under monochromatic light conditions, *srr1* mutants had a reduced sensitivity to red light specifically, but responded normally to both far-red and blue light (Fig. 1B–D). Because phytochrome B (*phyB*) is the major red light photoreceptor in *Arabidopsis*, we tested *srr1* for other typical *phyB*-mediated responses. *Arabidopsis phyB* mutations lead to pleiotropic phenotypes. *phyB* mutants are pale because of reduced chlorophyll accumulation (Reed et al. 1993). We measured chlorophyll content in red-light-grown seedlings and determined that *srr1* mutants accumulated intermediate levels of chlorophyll, between the levels of the wild type and a *phyB* null mutant (Fig. 2A). A saturating pulse of far-red light given at the end of the day induces several typical growth responses in *Arabidopsis*, including enhanced hypocotyl elongation (Robson et al. 1993; Aukerman et al. 1997). This end-of-day far-red (EOD FR) response is greatly diminished in *phyB* mutants (Fig. 2B; Robson et al. 1993; Aukerman et al. 1997), and *srr1* seedlings also displayed a reduced EOD FR response (Fig. 2B). Later in development, *phyB* mutants had longer petioles, a phenotype that was also observed in the *srr1* mutant (Fig. 2C). Moreover, *srr1* mutants had a subtle early-flowering phenotype in long days but flowered much earlier than the wild type in short days (Fig. 2D). The difference in flowering time between long and short days was therefore much smaller in *srr1* mutants than in the wild type (Fig. 2D). This suggests that *srr1* mutants, like *phyB* mutants,



**Figure 1.** *srr1* shows reduced sensitivity to white and red light. (A) Wild-type (Col-7), *srr1*, and *phyB-9* mutants grown in white light. (B) *srr1* mutants have longer hypocotyls in white and red light. Data are means  $\pm$  2  $\times$  S.E. of at least 12 seedlings for each light treatment. All seedlings were grown at 22°C in continuous light. Col (white bar), *srr1* (gray bar), and the appropriate photoreceptor mutants were grown for 6 d in 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white (*phyB-9*, black bar), 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  blue (*cry1*, hatched to the right bar), 15  $\mu\text{mol m}^{-2} \text{s}^{-1}$  red (*phyB-9*, black bar), or 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$  far-red (*phyA-211*, hatched to the right and left bar). (C) Fluence rate response curve in continuous red light of Col, *srr1*, and *phyB-9* seedlings. (D) Fluence rate response curve in continuous far-red light of Col, *srr1*, and *phyA-211* seedlings. (Open square) Col; (closed triangles) *srr1*; (open circles) *phyA-211*; (closed circles) *phyB-9*.

have a reduced sensitivity to day length (Fig. 2D; Reed et al. 1993; Blazquez and Weigel 1999). Taken together, these data strongly suggest that *srr1* mutants are defective in phyB-mediated signaling. To verify that *srr1* mutants had normal levels of phytochrome, we determined by Western blots that the levels of phyA and phyB were similar in the *srr1* mutant and the wild type (data not shown). These data suggest that *srr1* mutants affect phyB-mediated signaling rather than stability or biosynthesis of the photoreceptor.

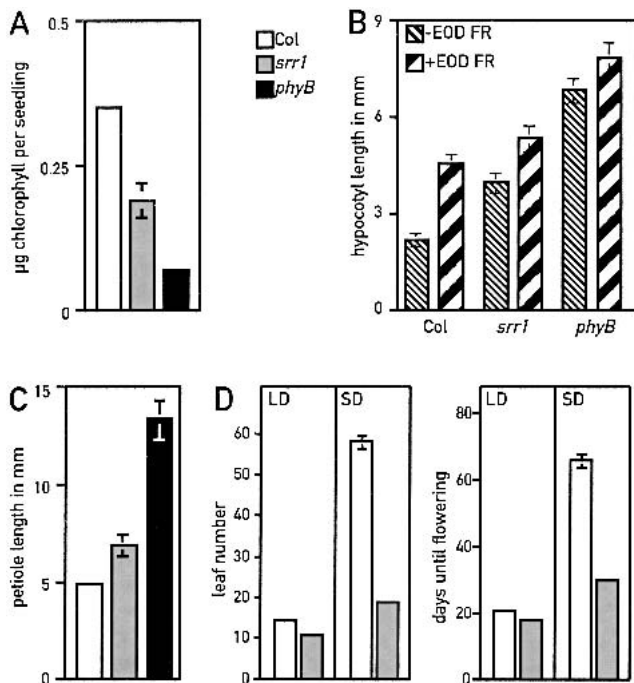
#### *SRR1* also acts independently of *phyB*

To verify that *SRR1* operates in the phyB pathway, we made double mutants between *srr1* and the null allele *phyB-9*. In red light (Fig. 3B), the *phyB srr1* double mutants had hypocotyls of the same length as the *phyB* single mutant. However, when de-etiolation was studied under white light, the *phyB srr1* double mutant had a stronger phenotype than the individual single mutants

(Fig. 3C). In white light, we also observed an additive phenotype for cotyledon expansion in the *phyB srr1* double mutant, which was not apparent in red light (data not shown). Collectively, these observations suggest that *SRR1* works in the phyB pathway, but also acts independently of phyB.

#### *SRR1* is required for multiple outputs of the circadian clock

*Arabidopsis* is a facultative long-day plant, flowering earlier in long than short days. The *srr1* flowering time phenotype suggests a defect in photoperiodic perception (Fig. 2D). A number of flowering-time mutants show a reduction or even an absence of sensitivity to day length (Mouradov et al. 2002). Such phenotypes are also observed in numerous circadian clock mutants; moreover, many of them also display an altered light sensitivity during seedling emergence (Mouradov et al. 2002). We therefore tested *srr1* mutants for circadian phenotypes



**Figure 2.** *srr1* and *phyB* mutants have several similar phenotypes. (White bar) Col; (gray bar) *srr1*; (black bar) *phyB*-9. (A) Chlorophyll content (expressed as nanograms of chlorophyll per seedling) of 7-day-old seedlings grown in constant 35  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  red light. Measurements were made in triplicate with 20 seedlings per sample. Values are means  $\pm 2 \times$  S.E. (B) The *srr1* mutant has a reduced end-of-day far-red (EOD FR) response. Seedlings were grown in control photoperiods (10 h light, 14 h dark, thin hatched bars to the right) or control photoperiods + 15 min FR light (thick hatched bar to the left). Values are means of 15 hypocotyls  $\pm 2 \times$  S.E. (C) The *srr1* mutant has longer petioles than the wild type. The petioles of 3-week-old plants grown in long days (16 h light, 8 h dark) were measured. Values are means of 16 petioles  $\pm 2 \times$  S.E. (D) *srr1* mutants flower early in short days. Long days (LD) are as in C; short days (SD) are 9 h light and 15 h dark. Values are the means  $\pm 2 \times$  S.E. with at least 18 plants for each condition.

by performing Northern blots of RNA from wild type and *srr1* mutants that were entrained in day–night cycles (LD) and released into constant light (LL). All circadian-regulated genes that were tested (*CAB*, *AtGRP7/CCR2*, *CCA1*, *CAT3*, *TOC1*, *GI*, and *FKF1*) showed shorter periods, more rapid dampening, and reduced amplitude of the oscillation in the *srr1* mutant background (Fig. 4A; data not shown). Quantification of the Northern blots revealed that the period of *AtGRP7/CCR2*, *CAB*, and *CCA1* oscillations was between 1.8 and 2.8 h shorter in the mutant than the wild type (data not shown). To test an unrelated clock-controlled phenotype, we recorded leaf movements in entrained *srr1* mutants that were released into constant light conditions. Again the *srr1* mutant had a period length of  $\sim 2$  h shorter than the wild type, 22.6 h in the *srr1* mutant versus 24.5 h in the isogenic wild type (Fig. 4B). These results, together with the reduced sensitivity to day length during photoperiodic flower induction (Fig. 2C), indicate that SRR1 function is

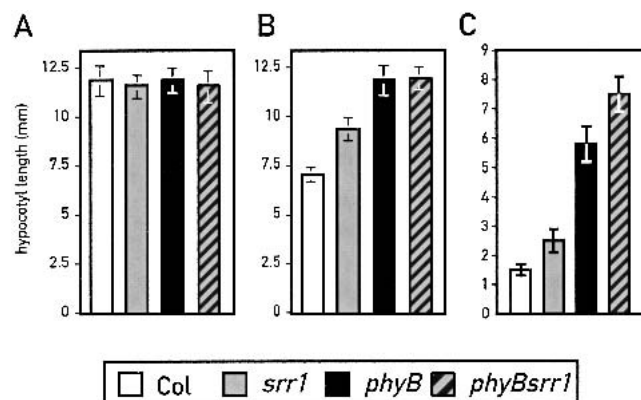
required for the timing of multiple outputs from the circadian clock.

#### *SRR1* function is required for clock-regulated gene expression during day–night cycles and in constant darkness

Experiments conducted in constant light cannot discriminate between a defect in the light input pathway and a defect in the oscillator (Covington et al. 2001). To test whether *srr1* is also required for clock function in the dark, we shifted entrained plants into constant dark (DD) conditions and tested the rhythmic expression of *CAB*, *CCA1*, and *CAT3* during one LD cycle and 2 DD cycles by Northern blotting (Fig. 4C). This experiment showed that during LD conditions, the peak of expression of genes such as *CAB* or *CCA1* is earlier in the *srr1* mutant than in the wild type (Fig. 4C). This indicates that under entrained conditions, the *srr1* mutants had a phenotype in circadian-regulated gene expression. *phyB* mutants also have an early phase of circadian gene expression (Hall et al. 2002; Salome et al. 2002).

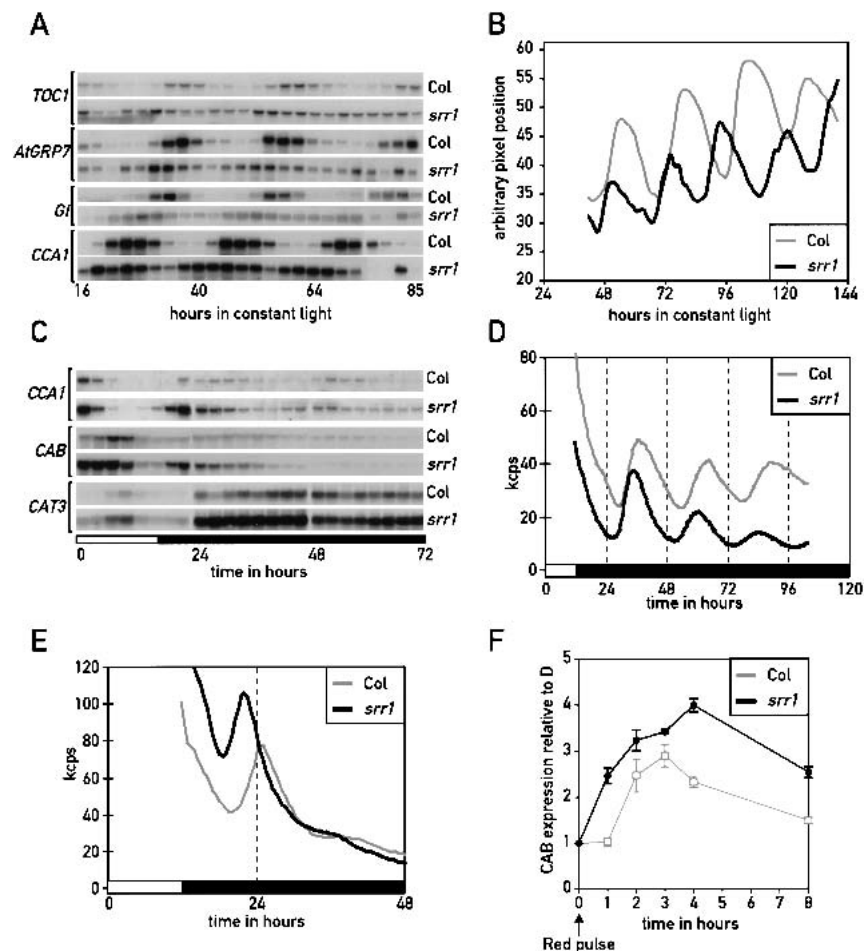
In *srr1* mutants, the early phase was also visible for *CCA1* transcript oscillation in DD, but the amplitude of rhythmic gene expression was not high enough to determine the period length in DD by Northern blotting. However, this experiment showed very clearly that in DD conditions, the transcript level of *CAT3* in *srr1* mutants stabilizes to a high steady-state level. This stabilization at a high level is dependent on *phyA* and *cry1* function but not *phyB* activity (Zhong et al. 1997). This observation is an additional piece of evidence linking SRR1 to *phyB* signaling rather than *cry1* or *phyA* signaling.

To further test for a possible function of SRR1 in DD conditions, we generated *srr1 CAB2::LUC* and *srr1*



**Figure 3.** SRR1 works in the *phyB* signaling pathway. (A) Hypocotyl length in the dark. (B) Hypocotyl length at 40  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  red light. (C) Hypocotyl length at 50  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  white light. Col (white bar), *srr1* mutants (gray bar), *phyB*-9 (black bar), and *srr1 phyB*-9 double mutants (hatched to the right bar with gray font). Data are means  $\pm 2 \times$  S.E. of at least 15 seedlings for each light treatment. All seedlings were grown at 22°C in continuous light.

**Figure 4.** The *srr1* mutation affects multiple outputs of the circadian clock. (A) *srr1* mutants affect cycling of multiple circadian-regulated genes in LL. Plants grown in long days were transferred at *zt*0 (dawn) to constant light at 20°C. Plants were harvested at 3-h intervals over 3 d, starting at subjective dusk (LL16) on the first day in LL (LL16 to LL85). The cycling of *TOC1*, *AtGRP7/CCR2*, *GI*, and *CCA1* was analyzed by Northern blotting. Actin was used as a loading control. (B) The *srr1* mutation affects the period length of leaf movement in LL-grown plants. (C) Circadian phenotype of *srr1* mutants in DD analyzed by Northern blotting. Plantlets were grown and analyzed as indicated in A, except that they were released into constant darkness. We also harvested samples during the last entrainment cycle. Northern blots were probed with *CCA1*, *CAB3*, and *CAT3*. (D) Circadian phenotype of *srr1* mutants analyzed with *CCR2::LUC* reporter lines. Col and *srr1* seedlings were entrained in LD (12:12) for 7 d before being released into DD and analyzed for bioluminescence. (E) Circadian phenotype of *srr1* mutants analyzed with *CAB2::LUC* reporter lines. Col and *srr1* seedlings were entrained in LD (12:12) for 7 d before being released into DD and analyzed for bioluminescence. (F) *srr1* mutants have an enhanced acute response. Col and *srr1* mutants were grown in the dark for 5 d on 1/2 MS 1.5% sucrose, then treated with a 3-min red light pulse at 50  $\mu\text{moles m}^{-2} \text{sec}^{-1}$  (LED light  $\lambda_{\text{max}} = 670 \text{ nm}$ ) and returned into darkness. The Northern blot was probed with a *CAB* probe. Hybridization signals were quantified with a phosphorimager and normalized with an actin probe. The average of 3 experiments  $\pm$  S.D. is shown.

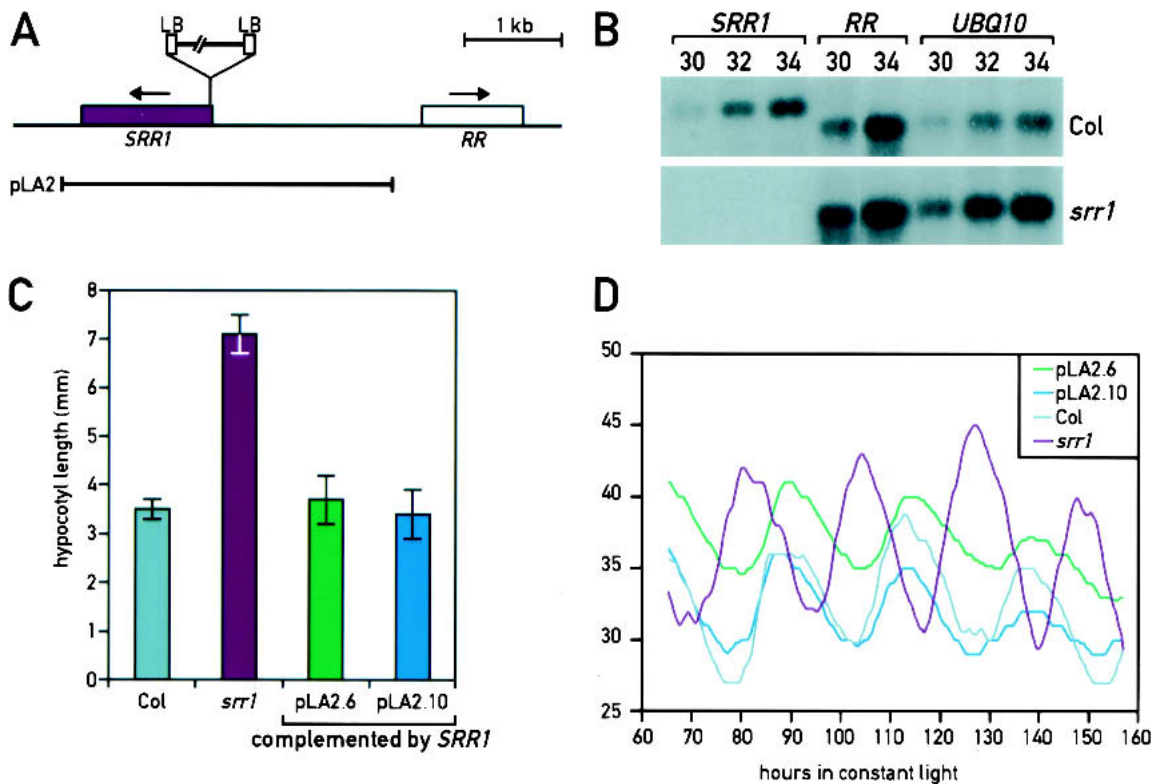


*CCR2::LUC* reporter lines. These plants were entrained in day–night cycles and released into constant darkness. These experiments showed that the phase of the circadian peak was 2 h earlier for both genes in the *srr1* mutant (Fig. 4D,E). For the *CCR2::LUC* line, the period length was shortened by 1.6 h in *srr1* mutants relative to the wild-type control (Fig. 4D). The rapid dampening of *CAB2::LUC* in DD prevented us from determining a period length of expression of this reporter gene. Taken together, these results suggest that SRR1 activity is required for normal oscillator function.

To test for a light-regulated gene expression phenotype when seedlings are transferred from the dark to the light without prior entrainment of the clock, we examined the induction of the *CAB* gene in etiolated seedlings in response to a pulse of red light. This early light induction of genes such as *CAB* is known as the acute response (Anderson and Kay 1995). Mutations in components of the *Arabidopsis* oscillator differentially affect this light response. *toc1-1* mutants have a normal acute response, contrasting with *cca1-1* and *phyB* seedlings, which show a reduced response (Somers et al. 1998b). The *srr1* mutants had an enhanced acute *CAB* response (Fig. 4F).

#### SRR1 codes for a nuclear/cytoplasmic protein that is conserved in numerous eukaryotes

The *srr1* mutant was identified in a collection of T-DNA-transformed *Arabidopsis* lines (Weigel et al. 2000). We crossed the mutant to an isogenic wild type and followed the segregation of the mutant phenotype and the T-DNA marker gene in >80 F<sub>2</sub> plants. The mutation was recessive for all phenotypes tested. There was a single T-DNA insertion site that cosegregated with the *srr1* mutant phenotype. We isolated genomic DNA flanking the left border (LB) of the T-DNA. The identified DNA matched the sequence of BAC F2O15 (nt 65296–65343), the insertion being in the 5'-untranslated region of a putative ORF coding for a protein of unknown function (F2O15.23, accession no. At5g59560). Using a combination of Southern blots and PCR, we determined that the T-DNA insertion is complex with two left borders (LB) flanking the insertion site (Fig. 5A). The insertion also resulted in the deletion of 82 nt. We identified and sequenced a cDNA coding for the gene that we call *SRR1* from an EST collection (GenBank accession no. AY127047). We tested the expression of the *SRR1* gene



**Figure 5.** *SRR1* gene structure and T-DNA insertion in the *srr1* mutant. (A) The region corresponding to BAC F2O15 (nt 63000–69000) is schematically represented. Genes in this interval are boxed, *SRR1* (in purple) and *RR* (a putative response regulator related to ARR1). The T-DNA insertion in the *srr1* mutant is represented; it has one left border (LB) on each side. The piece of genomic DNA used for complementation of the *srr1* mutant is indicated (pLA2). (B) The *SRR1* gene is not expressed in the *srr1* mutant. RT-PCR analysis of the two genes closest to the insertion site of the T-DNA in the *srr1* mutant (30, 32, or 34 PCR cycles were performed). *UBQ10* was used as a loading control. The number of PCR cycles performed before Southern blot analysis is indicated above each lane. (C) The *SRR1* gene complements the *srr1* mutation. More than 20 independent transformants of pLA2 in *srr1* rescued the visible phenotypes of *srr1*. Complementation of the *srr1* hypocotyl phenotype of two independent lines transformed with pLA2. (D) Two independent *srr1* plants transformed with pLA2 were analyzed for the period length of leaf movement in LL. Col is shown in light gray, the *srr1* mutant in purple, *srr1* pLA2.6 in green, and *srr1* pLA2.10 in blue.

and of the gene most proximal to the T-DNA insertion (a gene coding for a response regulator RR, the ARR1-like protein AB025604) by RT-PCR. We found that *SRR1* was not expressed in the *srr1* mutant, whereas expression of the *RR* gene was unaffected (Fig. 5B). To demonstrate that loss of the *SRR1* expression caused the *srr1* mutant phenotype, we complemented the mutant by transforming it with a piece of genomic DNA including 1.8 kb of promoter sequence and 0.5 kb of sequence 3' of the gene. Multiple independent lines were tested for de-etiolation and circadian phenotypes (Fig. 5C,D). These experiments confirmed that the lack of *SRR1* expression caused the phenotypes of the *srr1* mutant plants (Fig. 5C,D; data not shown).

Using BLAST and PSI-BLAST (Altschul et al. 1997), we found sequences homologous to the predicted *SRR1* protein in numerous eukaryotes including rice, mouse, human, *Drosophila*, and yeast (Fig. 6A). The function of these genes is presently unknown. A biochemical activity for these putative proteins is not immediately apparent because they contain no recognizable domains. A putative nuclear localization sequence is located be-

tween amino acids 26 and 30 of the *Arabidopsis* protein. This part of the putative protein is relatively well conserved between the animal and plant sequences (Fig. 6A,B). To test the subcellular localization of *SRR1* in *Arabidopsis*, we generated plants that expressed an *SRR1*-GFP fusion protein under the control of the Cauliflower Mosaic Virus 35S promoter. Fluorescence microscopy indicated that the *SRR1*-GFP fusion was found both in the nucleus and the cytoplasm of these plants (Fig. 6C). This fusion protein when expressed from its own promoter was capable of complementing the phenotype of the *srr1* mutants, indicating that the fusion to GFP did not alter *SRR1* activity (data not shown).

#### The *SRR1* transcript is induced by light

Numerous clock components are themselves under circadian regulation at the transcriptional level (e.g., see Wang and Tobin 1998). We tested this for *SRR1*, but found that steady-state *SRR1* mRNA levels were constant across the circadian cycle in LL (Fig. 7B). Because *SRR1* also plays a role in phytochrome-mediated light



## A

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Hs  . . . MAAAAAALLESQAAAPRRKR . RSAARRPRRREAAPRGREAAPRGREAAPRGPEAEFE . SDSGVVLRRRIWEAEKDLFI
Mm  . . . EPWSAVAPRRRKRAAGRRPRPGE . . . . . GPRAEPDADGEAVLRRRLREAEEDLRI
At  METTVKNSSSDGEWKVVLPSKGRQGRRRKPKPKG . . . . . QAEEEQPWKSDDLIDPQRQARL
Os  . . . . . MAAAAAGDWTVVRRRGRRRGDAAGDAASQ . . . . . PDAPPPSPSPRSPGPPRPPLPG
Dm  . . . . . MSNSGEDFQVVTRKKWMARKCLRRRDRH . . . . . KSESDYLIDCPDVNVEKFQPRL
Sp  . . . . . MDFIVVKRSRKKKNGRGK . . . . . FPKVSNITITQGDITLWS
Sc  . . . . . MDEFKASGTKFKKTGKKVVP . . . . . AKAF

Hs  SDFWSSALETIN . . RCLTKHLEQLKAPVGTLSDFGNLHLS . LPEESDVATDSIPREILVTGTCHLKVCYGIGNFA
Mm  SDFCSSALETIT . . ECLRKQLEQLQP . . . . . LTEALGRHLHGSSLPSAS . . . . . QEPLASSASHVKVCYGLGTFA
At  KQKMEISLKKIESSSFYTAFLEQLKS . . . . . PEVSN . . . . . QIRLVLGSETQLQMVMYIGISIE
Os  PRARLPPIRRPRRRHLPPIRRAP . . . . . PPPGIP . . . . . AAAPLPSLAPSGLSLLGVGSFE
Dm  ENLCTEMCQSDYFLVAMEALQQLEG . . . . . . . . . . . IRKPLERIVCLGLGPFS
Sp  QDTLQRRKLDNSREKLEYSEFLKSVQS . . . . . . . . . . . QLSEFKDPIHKIILGLGRIH
Sc  EEIVQTNRDVLLKSAFLSDDLLENLQP . . . . . . . . . . . HLNNIKKIRCAVIGNFK

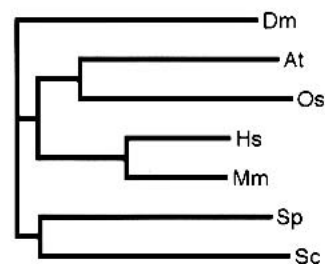
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Mm  SCPTARIQLAFMLLFLEKQVPR . . . . . SHCWVYDPLFSQTEVSVLTSLG . VTVLSENEEGKRSVQGQPTVFYMPHCGTA
At  SYESPRFQLSIALIMKREFDWVG . . . . . DNIEVFDPLVSATSSYLES LG . CSVLSVNEQARR . EALKPTLFFMPCHEAN
Os  NSPSSRLQALALALREDLLLPSSAHADLFDPVLSAAECAAAAALG . FTVPGVNDGCR . RADEPTLFYMPHCEAS
Dm  RTYHALHCAAFVIGLHRHHKIRE . . . . . ALYFDPVFRDSEKELIRLFD . GCIMSKDCAGKH . EATVPTLYYLPHCPYA
Sp  T . LTASLQLSLFFEIFMIFNLQP . . . . . RFCSFYDPAFLKDDVEFLENKG . FCVLP . . . . . DPPTPSCLKYTLLYMPHCPTS
Sc  EDFPATYCFALLLEITDYIKSEDERDVVVS LYDPIFTKEELQYKSLGSKWLIIEEFSENDAIDYESVLYFLPHAPLD

Hs  LYNNLLWSNWS . VDALSKMVIIGNSFKGLEER . . . . . LLARILQKNYPYIAKILKGLEELEFPQTSQYMD . IFN
Mm  LYNNLLWSNWS . ADALSRVLIIGNSFKGLEER . . . . . LLARILQENYPYIAKVS DRIAGPGF . . . . .
At  LYSNLLQANWR . MDRLSKIALFGNSFQMYEEQV . . . . . SFDAEVICATKRIIAAQRVTSEFAIETESDDYFA . AFH
Os  LYDALLAANWEPPSQLRHV CVLGNSFRNYAIAEENRSGPAARAKHVLAERFAWEERVSEKGGVDDDDDDVFNRFAFN
Dm  LMHNILWSNWK . RETLPNVFLISNSFEMLTMT . . . . . RNQDDHITRIVEHCTETPLEDDYEHNN . VFN
Sp  LYETWLAAYVN . . . . . DDPHFIMCGNNLQLYVDNK . . . . . PSKEIVSTYPNVYKMCTKNYYTRLLFPFEPNVYAFN
Sc  LTENILSSQRPHLWLANNMISHTDRYTKAKLCEN . . YPNLSKLVHYLQTNAPPEVKKAHADVDFATFIPKRKRKNRN

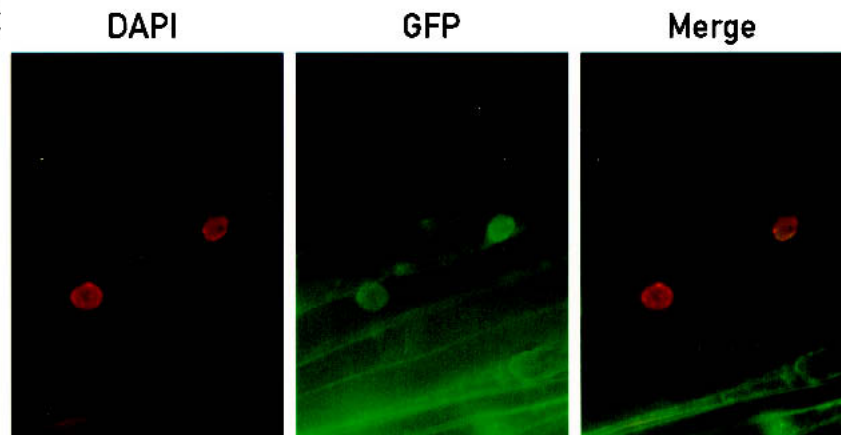
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Mm  . . . . .
At  DSSWHFFSSGIDSELPFLVSD . . . . .
Os  ETSWHFFEVDAAADLAAAVASTGGRR . . . . .
Dm  DLSLHTFPQESLPGSNDEAFWTRCAPLKVNEDELITETETSLAALKLESE . . . . .
Sp  DLSFHFDHDAQSFVEKKQPSKFVDASSVAEHPS . . . . .
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## B

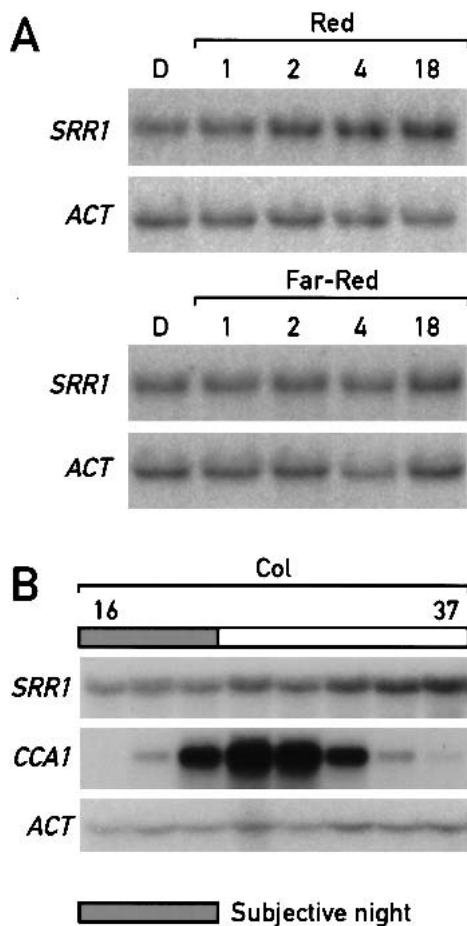


## C



**Figure 6.** SRR1 is localized in the nucleus and the cytoplasm, and homologous proteins are found in numerous eukaryotes. (A) The alignment was generated using the ClustalW program (<http://www2.ebi.ac.uk/clustalw>). The sequences used are *Arabidopsis* SRR1 cDNA (accession no. AY127047); a mouse cDNA clone (accession no. AK012640.1); a human putative protein predicted from the genomic sequencing program (accession no. Z99714.2); a *Drosophila melanogaster* putative protein predicted from the genomic sequencing program (accession no. AE003721.1); a *Schizosaccharomyces pombe* putative protein (accession no. AL022305.1); the *Saccharomyces cerevisiae* hypothetical protein YLR412w (accession no. S59378); and a sequence from the rice genome (<https://portal.tnri.org/rice/p/UserHome>) that was found to have the highest homology to *Arabidopsis* SRR1 (Goff et al. 2002). Identical residues are boxed in green; red shading indicates residues identical in at least 4/7 sequences, similar residues in at least 4/7 sequences are shaded in blue. (B) Phylogenetic relationship between various SRR1 homologs. (C) DAPI fluorescence, GFP fluorescence, and merge picture of Col transformed with a SRR1-GFP fusion construct (CF325).





**Figure 7.** The mRNA level of *SRR1* is light-regulated, but is not under clock control. (A) *SRR1* mRNA is up-regulated in response to red light but not far-red light. Five-day-old etiolated seedlings grown on 1/2 MS 1.5% sucrose were shifted into red light or far-red light for the indicated amount of time; the Northern blot was probed with *SRR1*- and *actin*-specific probes. (B) *SRR1* mRNA levels don't cycle in LL. The samples were as in Figure 4A corresponding to LL16 to LL37. The blot was probed with *SRR1*, followed by *actin* as a loading control and *CCA1* as a cycling control.

signaling, we tested whether the *SRR1* message was up-regulated in response to light. The steady-state level of *SRR1* mRNA was induced when dark-grown seedlings were transferred into red light. The induction was already visible 2 h after the shift into the light (Fig. 7A). Interestingly, the *SRR1* transcript was not modulated by far-red light (Fig. 7A). This pattern of light induction is consistent with a role for *SRR1* in phyB signaling.

## Discussion

In *Drosophila*, CRY plays a role both in the light input pathway to the clock and in the core oscillator (Krishnan et al. 2001). Similarly, the detailed characterization of numerous *Arabidopsis* mutants makes it increasingly apparent that it is not simple to distinguish between

mutants in the circadian system and photomorphogenic mutants (Devlin and Kay 2001). A common feature among these mutants is that they are affected in the photoperiodic induction of flowering, light sensitivity during seedling emergence, and clock-controlled gene expression. It should be noted that not all light-signaling mutants have major clock phenotypes and certain clock mutants de-etiolate quite normally. Photoreceptor mutants such as *phyB* show dramatic de-etiolation and flowering time phenotypes, but they have mild period length phenotypes of clock-controlled genes (Somers et al. 1998a). This has been demonstrated by using a *phyAphyBcry1cry2* quadruple mutant that is practically blind to white light, but still shows robust cycling of leaf movements in constant light (Yanovsky et al. 2000). In contrast, the *toc1-1* mutant shows normal de-etiolation phenotypes but has dramatic circadian defects (Somers et al. 1998b).

### Mutants affecting the circadian system are often impaired in de-etiolation in red light

The characterization of the *srr1* mutant demonstrates a role for *SRR1* both in phyB signaling and as an important regulator of the circadian clock. *srr1* mutants are affected in hypocotyl elongation, greening, the end-of-day far-red response, petiole growth, and flowering in a manner very similar to *phyB* mutants (Figs. 1, 2). In addition, dampening of *CAT3* transcript oscillations to a high level in DD is not affected in the *srr1* mutant (Fig. 4C). This result is interesting in view of the fact that this stabilization to a high level requires both phyA and cry1 but not phyB, reinforcing the notion that *SRR1* works specifically in a phyB signaling pathway (Zhong et al. 1997). Moreover, *srr1* and *phyB* mutants have an early phase of circadian gene expression (Fig. 4; Hall et al. 2002; Salome et al. 2002).

It is quite intriguing to note that *ztl*, *elf3*, and *gi* mutants all have a specific deficiency for de-etiolation in red, but not far-red or blue light (Huq et al. 2000; Reed et al. 2000; Somers et al. 2000). The same is true for *srr1* mutants (Fig. 1). Our analysis indicates that similarly to GI and ELF3, this is caused by decreased phyB-mediated signaling. The analysis of double mutants reveals that *SRR1*, like ELF3, affects both phyB-mediated signaling and a pathway working in parallel with phyB (Liu et al. 2001). Hypocotyl elongation in the dominant *lhy* mutant and plants overexpressing *CCA1* also show decreased sensitivity to white light, but it is not known whether this is restricted to one monochromatic light condition (Schaffer et al. 1998; Wang and Tobin 1998).

### A role for *SRR1* in phyB signaling

A number of mutants that define components in phyB-mediated signaling have been identified in recent years. The signaling events occurring after photoperception appear rather complex. A number of those components, including ELF3 and GI, have been shown to be nuclear-

localized. The best understood branch of phyB-mediated signaling involves the bHLH transcription factors PIF3 and PIF4. PIF3 and PIF4 interact with phyB in a conformation-dependent manner (Martinez-Garcia et al. 2000; Huq and Quail 2002). In the case of PIF3, the interaction with phytochrome occurs when the transcription factor is bound to light-regulated promoters (Martinez-Garcia et al. 2000). Because phyB is translocated into the nucleus in response to light, this would potentially give a very short light-signaling cascade (Smith 2000). This picture contrasts with the fairly large number of mutants implicated in phyB-mediated signaling, and is likely to represent one facet of this complex network (Hudson 2000). The exact function of SRR1 in phyB-mediated light signaling is presently not known. We have no indication for a direct interaction between SRR1 and the phytochromes (data not shown). Double-mutant analysis with other phyB signaling mutants could shed some light on this. In addition, it will be interesting to see if the subcellular localization of phyB is affected in an *srr1* background, as SRR1, like the phytochromes, can be found both in the nucleus and the cytoplasm (Fig. 6C).

#### *SRR1 and ELF3 are important for distinct aspects of the circadian clock*

The comparison of *srr1* and *elf3* mutants shows that they have both similar and distinct phenotypes. The circadian phenotype of *srr1* mutants in LL is less dramatic than the one observed for *elf3-1* null mutants (Reed et al. 2000). This is not caused by allele strength, because *srr1* is a null allele (Fig. 5B). *srr1* mutants clearly have a short period phenotype in LL, and dampening of clock-controlled genes is more rapid than in the wild type (Fig. 4). *srr1* and *elf3-1* mutants also show an early phase of circadian gene expression in LD and an enhanced acute response for CAB expression in response to light (Fig. 4; Hicks et al. 1996; Covington et al. 2001). Moreover, ELF3 works in part in the phyB pathway as indicated by the phenotypic analysis of the mutant and the direct interaction of phyB and ELF3 in vitro (Liu et al. 2001). ELF3 has been shown to control the light input pathway to the clock. *elf3* mutants show arrhythmia in the light but remain rhythmic in the dark (McWatters et al. 2000). This contrasts with *srr1* mutants, which have a circadian phenotype both in the dark and in the light (Fig. 4). Therefore, despite certain similarities between *srr1* and *elf3* mutants, SRR1 activity is required for normal oscillator function in contrast to ELF3, which is required for light input to the clock specifically.

#### *SRR1 is required for normal circadian oscillator function*

Single null mutations can unconditionally abolish overt rhythms in some species, indicating that the cognate, wild-type genes are required for clock function (Young and Kay 2001). No such recessive, arrhythmic single mutant has been reported in *Arabidopsis* (Green and Tobin

2002). In this regard, the *elf4* mutant has the most dramatic phenotype, but *elf4* still retains low-amplitude cycling (Doyle et al. 2002). *srr1* null mutants display rhythmicity in DD or LL but with an early phase and a shorter period (Fig. 4). These data suggest that there is redundancy at the level of SRR1 or that SRR1 activity is required for proper clock function rather than being a key element of the clock itself. The circadian phenotype of *srr1* mutants, like the one reported for *toc1*, *cca1*, or *lhy* loss-of-function alleles, results in a shortening of the period length in constant conditions (Green and Tobin 1999). *toc1-2*, a putative null allele, causes dampened rhythms with a short period in constant light (Strayer et al. 2000). The dampening of the oscillation does not seem to occur in *cca1* loss-of-function mutants (Green and Tobin 1999). This relatively mild phenotype of *cca1-1* is caused by the redundancy of CCA1 and LHY (Alabadi et al. 2002). Interestingly both *cca1-1* and *lhy* loss of function mutants have flowering-time phenotypes specifically in short days. The double mutant is almost day neutral and has an early phase of circadian gene expression during day-night cycles (Mizoguchi et al. 2002). It has been proposed that this circadian phenotype affects the phase of *CO* expression during short days, thereby leading to early flowering (Mizoguchi et al. 2002; Roden et al. 2002; Yanovsky and Kay 2002). Interestingly, the circadian and flowering-time phenotypes of *srr1* mutants are very similar to those of *cca1*, *lhy*, and *toc1* loss-of-function alleles, clearly suggesting a role for SRR1 in the clock (Figs. 2, 4; Strayer et al. 2000).

Mutants affecting clock components have distinct phenotypes for the acute light response of *CAB* gene expression. *cca1* mutants affect both the phytochrome-mediated acute response and the circadian peak of *CAB* expression (Green and Tobin 1999). Similarly, *srr1* mutants show a phenotype during light induction of *CAB* expression (Fig. 4E). The enhanced acute response phenotype observed in the *srr1* mutant was also reported for *elf3*, a mutant in the light input pathway to the clock (Covington et al. 2001). This contrasts with the situation in *toc1-1*, which affects the period of light-regulated genes without effects on the acute light response of *CAB* expression (Somers et al. 1998b).

#### *A circadian clock function for SRR1 homologs in animals?*

Many extremely diverse organisms have a circadian clock. In the systems in which it has been studied, the mechanism generating an ~24-h period appears to be conserved mechanistically; however, the components of the machinery are mostly unrelated (Young and Kay 2001). The cryptochromes are an exception in this context because they are important for circadian regulation both in plants and animals (Cashmore et al. 1999). Their function, however, is not identical in different species, and it has been proposed that they have arisen twice during evolution (Cashmore et al. 1999). Cloning of *SRR1* in *Arabidopsis* identifies a putative protein of unknown biochemical function but with clearly related

proteins found in numerous eukaryotes. Despite the involvement of phytochromes in the light input pathway to the clock in cyanobacteria, we have not found any clear *SRR1* homolog in prokaryotes (Schmitz et al. 2000).

In the context of circadian biology, the putative homologs in human, mouse, and *Drosophila* are of particular interest. It must be pointed out that in most organisms these genes are only described based on annotation of sequenced genomes. Because there are ESTs (expressed sequence tags) in mouse and given the good conservation between mammals, it is likely that the human equivalent is a functional gene. The mouse *SRR1* homolog maps in proximity to the *FRP2* short-period circadian rhythm QTL (quantitative trait locus; Shimomura et al. 2001). This is noteworthy in light of the fact that among the numerous mouse QTLs affecting the circadian clock, only two have a short period, and one of them, *FRP2*, maps close to the mouse *SRR1* homolog. Cloning this gene is one way to address the possible conservation of *SRR1* function across species. The mouse *SRR1* homolog is expressed at very low levels in all tested tissues with no circadian oscillation of its mRNA levels (S.A. Brown and U. Schibler, pers. comm.). The same is true for *SRR1* in *Arabidopsis*; however, this is not exceptional for factors required for proper clock function (Fig. 7; Sugano et al. 1999; Somers et al. 2000). We have not found an *SRR1*-related protein in *Neurospora*, but given that the entire genome has not been sequenced yet, this does not exclude the existence of a related protein in this species. More distant relatives of *SRR1* have also been found in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* with significantly better homologies between *SRR1* and the *S. pombe* sequence than between *SRR1* and YLR412w from *S. cerevisiae*. There are a few reports on circadian rhythms in yeast (Kippert and Hunt 2000). The yeast genes are of unknown function, and systematic yeast two-hybrid analysis performed with YLR412w has not been informative concerning a putative function of this protein in *S. cerevisiae*. Despite a lack of conservation of the elements that constitute a central oscillator in animals, fungi, and plants, there are some common features such as regulated nuclear translocation, phosphorylation, and transcriptional-translational negative feedback loops (Young and Kay 2001). *SRR1* might modulate one of those processes.

## Material and methods

### Plant material and light sources

The progeny of 2600 independent *Arabidopsis* ecotype Col-7 transformants (Weigel et al. 2000) were screened in 50  $\mu$ moles  $m^{-2} sec^{-1}$  white light. We screened ~10 T2 seeds per original transformant. Seeds were surface-sterilized and plated on Petri dishes on 1/2 MS, 0.7% phytagar. Plates were stored in the dark at 4°C for 3 d and moved into constant white light at 22°C. For monochromatic light conditions, germination was induced by a white light treatment; the seedlings were then grown in the appropriate light conditions for 4–6 d depending on the experiment. Experiments in monochromatic light were performed in

a Percival E-30LED using either the blue ( $\lambda_{max}$  = 469 nm), red ( $\lambda_{max}$  = 667 nm), or the FR ( $\lambda_{max}$  = 739 nm) diodes, at 22°C in continuous light. Light intensities were determined with a Li-Cor LI-1800 spectroradiometer or with an International light IL1400A photometer equipped with an SEL033 probe with appropriate light filters.

### Petiole-length and flowering-time determination

Petiole length was determined as described (Aukerman et al. 1997). Flowering time was measured according to Blazquez and Weigel (1999). Briefly, seeds were stratified at 4°C for 3 d, and plants were grown at 23°C in long (16 h light, 8 h dark) or short (9 h light, 15 h dark) days under a mixture of 3:1 cool-white and Gro-Lux fluorescent lights.

### Hypocotyl length and chlorophyll accumulation

Measurements of hypocotyl length were performed as described (Neff and Chory 1998). For chlorophyll determination, triplicates of 20 seedlings were each incubated in 1 mL of 80% acetone overnight in the dark at room temperature under constant shaking. The OD of the solution was determined at 647 nm and 663 nm, and the chlorophyll content was calculated with the following formula: micrograms per microliter of total chlorophyll =  $18.71 \times (OD_{647}) + 7.15 \times (OD_{663})$ . The effect of the end-of-day far-red treatment was determined as in Aukerman et al. (1997).

### Plant DNA and RNA preparation

Plant DNA was prepared either according to Neff et al. (1998) or with Plant DNaZol Reagent (GIBCO-BRL) according to the manufacturer's instructions.

RNA was extracted as described (Heintzen et al. 1997). We ran formaldehyde MOPS gels loaded with 10–15  $\mu$ g of total RNA per lane and transferred the RNA with 10 $\times$  SSC. Probes generated by random priming were described in Leutwiler et al. (1986). Northern and Southern blots were hybridized with Church buffer at 62°C and washed according to the manufacturer's instructions. For quantification, we used a PhosphorImager, Imagequant (Molecular Dynamics). RNA levels for clock-regulated genes were normalized to the levels of actin RNA. Normalized data were analyzed using FFT-NLLS, to provide objective period estimates. This software returns a valid best-fit period from such short and sparse time series, although the associated estimates of uncertainty of fit must be treated with caution.

### Cloning of the *SRR1* gene

Because the *srr1* mutant came from a T-DNA insertion collection, we determined that the phenotype cosegregated with the T-DNA insertion marker by analyzing 80 F2 progeny of a cross between the homozygous *srr1* mutant and wild-type Col-7. The *srr1* phenotype is recessive, and the original mutant contained a single T-DNA insertion site. For subsequent experiments the mutant was back-crossed twice.

To obtain DNA sequence flanking the T-DNA insertion site, we used a PCR technique related to the Genome Walker Kit (Clontech). Sequencing identified the T-DNA left border sequence flanked by 70 bp of sequence of unknown origin and 48 bp of BAC F2O15 (nt 65296–65343), finishing with an *HindIII* site ligated to the adaptor sequence. The insertion site was then confirmed by PCR with various primers. Using Southern blots, we determined that the insertion is complex, with the other end of the T-DNA also containing a Left Border as determined by

PCR followed by sequencing. The other end flanks nt 65426 from BAC F2O15, and the T-DNA insertion therefore deletes 82 nt corresponding to nt 65344–65425 of BAC F2O15. This T-DNA insertion is just upstream of a putative ORF for which we found the following EST clones: SQ058e07F (AV557017) and SQ091c08F (AV558172; Kasuza DNA Research Institute). The longer of the two, SQ091c08F, encompasses nt 64054–65328 of BAC F2O15 including a putative ORF identified by the *Arabidopsis* genome project.

#### RT-PCR

For the analysis of transcript levels, a reverse transcription reaction was performed with total RNA extracted from wild-type and *srr1* mutant plants. An oligo(dT)<sub>(12–18)</sub> primer was hybridized to 1 µg of RNA. The reverse transcription was performed with Superscript II (GIBCO-BRL) according to the manufacturer's instructions. After the RT reaction, the cDNA was diluted threefold with dH<sub>2</sub>O, and 1 µL of diluted cDNA was used for PCR reactions with gene-specific primers for *UBQ10*, *SRR1*, and *RR*. PCR reactions were stopped after 30, 32, and 34 cycles, and the reactions were analyzed by Southern blotting. To test for DNA contamination, a mock RT reaction was performed in the absence of oligo(dT)<sub>(12–18)</sub>. The product was then PCR-amplified and Southern blotted as described above.

#### Complementation of the *srr1* mutant

For complementation of the *srr1* mutant, we gel-purified the 3.4-kb *SpeI* fragment from BAC F2O15 encompassing the *SRR1* gene (nt 63753–67173). The fragment was ligated into an *XbaI*-digested and phosphatase-treated pPZP211 vector (Hajdukiewicz et al. 1994). The resulting construct (pLA2) was introduced into *Agrobacterium tumefaciens*, strain GV3101. *srr1* plants were transformed with the spray method (Weigel et al. 2000). T2 seeds from multiple independent transformants were used to confirm the complementation.

#### Microscopy

Using standard cloning techniques, we fused a modified GFP (Friedrichsen et al. 2000) in frame after the last codon of the *SRR1* gene. This fusion gene was either expressed under the 35S promoter by cloning it into pCHF3 (Jarvis et al. 1998) to create pCF325, or expressed from its own promoter to create pCF327. Col plants transformed with CF325 and *srr1* mutants transformed with pCF327 were generated as described above. We performed microscopy with 5-day-old dark-grown homozygous single-insertion Col pCF325 plants. The seedlings were mounted in 20% glycerol, 1/2 MS, and 0.5 µg/mL DAPI (Sigma) and were observed with a DAPI filter or a narrow band GFP filter (Chroma 41020) on a Zeiss Axioplan 2 fluorescence microscope. Images were recorded with a Quantix CCD camera and pseudocolored using Photoshop.

#### Analysis of circadian phenotypes

Leaf movement rhythms were monitored in a Kujata imaging system and analyzed using FFT-NLLS as described (Swarup et al. 1999). Experiments comparing *srr1* with Col-7 were repeated three times, with very similar results. Figure 4 presents data from a representative experiment. Leaf movement rhythms were analyzed in a single experiment for two *srr1* mutant lines that were independently transformed with the complementing construct, pLA2. Seedlings were arranged randomly with respect to genotypes within each experiment, to avoid positional

bias in the imaging arrays. Transcriptional rhythms of *CAB2* and *CCR2* expression were assayed using luciferase reporter gene fusions as described (Doyle et al. 2002). The *CCR2::LUC* and *CAB2::LUC* constructions were transformed into Col-7 and the *srr1* mutant (Weigel et al. 2000), and progeny from three independent transformants were analyzed for each genotype/transformation combination. Rhythmic data on the T2 and T3 generations were collected on a TopCount (Packard). Period estimates were generated using FFT-NLLS, as described above.

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